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IDENTIFICATION OF *LACTOBACILLUS PLANTARUM* STRAINS USING RAPID MOLECULAR TECHNIQUES

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ABSTRACT

The study was under taken to identify *Lactobacillus plantarum* stains at molecular level by using PCR and RAPD. The genus and species specific PCR primers for *Lactobacillus plantarum* was used for the identification of the probiotic cultures. The randomly amplified polymorphic DNA (RAPD) technique was used to produce potential strain-specific markers to differentiate the two stains of *L.plantarum*. The RAPD technique is a PCR-based discrimination method in which short arbitrary primers anneal to multiple random target sequences. The genus specific PCR assay conducted using the template DNA resulted in an amplification of 250 bp PCR product and species specific PCR resulted in 248 bp amplification. The random primer OPAP-01 was used for RAPD PCR. The banding patterns obtained with the two cultures were quite distinct and good enough to discriminate them as two separate strains. Molecular level identification methods are a powerful alternative to the conservative differentiation of bacteria by plating. These techniques will certainly prove very useful when studying the presence of probiotic strains *in vivo*.

KEY WORDS: **Lactobacillus, PCR, RAPD, Probiotics.**

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INTRODUCTION

Probiotic are live microbial feed supplements that are used increasingly in dairy products. Confusion due to false declarations and the uncritical selection of strains is a major problem in probiotic research. Efficacy of probiotic cultures depends on special properties of single strains, however. The strains of the genera *Lactobacillus* and *Bifidobacterium*, especially out of the groups of *Lactobacillus acidophilus*, *Lactobacillus casei, Lactobacillus plantarum* and *Lactobacillus reuteri/fermentum* collected from commercial probiotic food samples as well as from pharmaceuticals and nutrients for animals should be compared with reference strains from culture collections. Phenotypic criteria reveals a group of isolates at genus level and, in most cases, a correct identification of species can be attained through genotypic characterization. False declarations of cultures used world-wide as probiotics could thus be detected. In order to confirm questionable species or to characterise specific strains within a species, molecular-based methods had to be applied.(Reuter *et al* 2002) Administration of probiotics can prevent or treat certain conditions including acute diarrhea, symptoms associated with irritable bowel syndrome, infection after surgery, certain cancer, high serum cholesterol and diabetes. Common probiotics include species of the genera *Lactobacillus, Bifidobacterium* and *Saccharomyces boulardii*. Lactobacillus species play a major role in fermented dairy products and also contribute to the therapeutic aspects of human health. Among these *Lactobacillus plantarum* is a flexible and versatile microorganism that inhabits a wide variety of environmental niches, including the human gastrointestinal tract (Kandler & Weiss, 1986). But the mechanisms by which probiotics influence the host organism are only beginning to be explored. To fully understand the beneficial effects of probiotics, it is important to identify and characterize the intestinal microbial community. As the probiotic capacities are strain-dependent, methods for reliable identification of lactobacilli at the strain level are of great importance, especially for the quality control of approved strains to avoid health risks, misleading and for the description of new strains. Nowadays, the main focus for the identification has moved from phenotypic to genotypic methods as they yield more sensitive and accurate results. Hence, rapid methods are often important for quality control and *in vivo* identification of probiotic strains and species. The main aim of the research is identify the potential probiotic lactic acid bacteria (LAB), *Lactobacillus plantarum* strains, which could be used in dairy products

MATERIALS AND METHODS

Lactobacillus plantarum Lp91 (MTCC 5690; Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India), the subject of this study, was a laboratory isolate of human origin and *Lactobacillus plantarum* Lp5276 (also designated as CSCC5276, NCDO82 or VTTE-71034) used as a reference strain in this study was procured from Dr. N. P. Shah from Victoria University, Australia. The purity of the *Lactobacillus* cultures was ascertained by Gram staining and microscopic examination. The test and reference were propagated and maintained in MRS (de Man–Rogosa–Sharpe broth, HiMedia, India) broth at 37°C for18 h. The active bacterial cultures were maintained in litmus milk (4^0C) and also as glycerol stocks (- 70°C).

DNA isolation

For the identification of lactobacillus cultures by PCR, the genomic DNA from the cultures were extracted by Pospiech and Neumann (1995) method. The cells were harvested from 1.5 ml of overnight grown culture in a 2.0 ml microcentrifuge tube at 10,000 rpm for 10 min. to pellet the cells. The supernatant was decanted and the pellet was resuspended in one ml of autoclaved Milli Q water. The pellet obtained from 1.5 ml of respective cultures were resuspended in 0.5 ml SET buffer containing freshly prepared lysozyme at a concentration of 1 mg/ ml. The above step was followed by addition of 1/10th volume of SDS and addition of, one third volume of 5M NaCl and one volume of phenol: chloroform: isoamylalcohol (25:24:1) to the above mix

which was incubated at room temperature for 30 min. with frequent inversions. After which centrifugation at 4500 rpm/ 15 min. was done and the aqueous phase was transferred to the new tube. The DNA was precipitated by addition of one volume of isopropanol. After centrifugation at 12,000 rpm for 10 min. the pellet obtained was washed with 500 µl of 70% ethanol and vacuum dried in a Speed Vac.System (Martin Christ, Germany). The DNA pellet was finally dissolved in 50 µl of autoclaved Milli Q water or TE buffer (pH 8.0) and stored at -20°C until further use.

Genus and species specific PCR

Genus specific primer for Lactobacillus, LbLMA1 /R 16 - 1 (sequence of primers : 5'ctc aaa act aaa caa agt ttc 3'/ 5'ctt gta cac acc gcc cgt ca3') with product size 250 bp was used. The reaction mix, comprising of 10X PCR buffer (containing MgCl2), dNTPs and primers were prepared and distributed to reaction tubes according to the requirements. The final volume of the PCR mix was adjusted to 25µl. Amplification was carries out as initial denaturation at 95 °C for 5 min, followed by 35 cycles consisting of denaturation at 95 °C for 30 sec, annealing at 58^{o} C for 30 sec, and extension at 72 °C for 30 sec, and a final 7 minute extension step at 72 °C. PCR amplified products obtained with different templates were electrophoresed on the agarose gels (1%). Species specific primer for *L.plantarum,* Lpla2/Lpla3 (sequence of primers: 5'cct gaa ctg aga gaa ttt ga 3'/ 5' att cat agt cta gtt gga ggt 3') with product size 248 bp was used. The reaction mix, comprising of 10X PCR buffer (containing MgCl2), dNTPs and primers was prepared and distributed to reaction tubes according to the requirements. The final volume of the PCR mix was adjusted to 25µl. Amplification was carries out as initial denaturation at 95 °C for 5 min, followed by 35 cycles consisting of denaturation at 95 °C for 30 sec, annealing at 60^{o} C for 30 sec, and extension at 72 °C for 30 sec, and a final 7 minute extension step at 72 °C. PCR amplified products obtained with different templates were electrophoresed on the agarose gels (1%).

RAPD profiles of Lactobacillus cultures

PCR with random primer namely OPAP-01 (5' aactggcccc 3') was carried out. PCR reaction was performed in 25 µl reaction volume containing 2 µl of genomic DNA, 2.5 µl PCR buffer, 2.0 µl of oligo primer (OPAP-01), 2.0 µl of 200 µM dNTP and 0.5 µl of 1.0 U Taq DNA polymerase. Amplification was carries out as initial denaturation at 95 °C for 5 min, followed by 45 cycles consisting of denaturation at 95 °C for 30 sec, annealing at 37-40 ^{o} C for 30 sec, and extension at 72 °C for 2 min, and a final 10 minute extension step at 72 °C. The PCR products were electrophoresed on 1.5 % agarose gel with ethidium bromide (0.5 µg/ ml) (Amersham Biosciences, USA) at 100 V using 1X TAE buffer. Gels were monitored on UV transilluminator. The gels were subsequently photographed (MiniBis BioImaging System, UK).

RESULTS AND DISCUSSION

Genus and species specific PCR

After ensuring the purity of the indigenous probiotic the PCR assay conducted with the template DNA obtained from the *Lactobacillus* cultures resulted in the amplification of a 250 bp PCR products on the agarose gel which was specific for lactobacilli only. On the basis of our PCR results, *Lactobacillus plantarum* 91 and *Lactobacillus plantarum* 5276 showed the positive signal in the form of a distinct 250 bp band on the gel **.** Our results in this regard are in complete agreement with those of Dubernet *et al*, 2002; Suja, 2003 and Neelakanteshawara, 2005 who also demonstrated amplification of 250 bp product in the PCR assay with all the standard cultures as well as wild isolates of lactobacilli used in their study as well as the cultures used in this study. After ascertaining the test cultures as *Lactobacillus*, we directed our efforts to identify the culture at species level by using Lpla3/Lpla2 set of primers which is specific for *L. plantarum*. The PCR assay conducted with the template DNA resulted in an amplification of 248 bp PCR product. (Fig.1) The PCR assay was shown to be specific for *L. plantarum* since other bacterial species, including several species within the closely related Lactobacillus genus, were not detected with the PCR primers used. Dickson *et al* 2005 have observed that species specific PCR can be used as a reliable method for the identification *L.fermentum* species in various oral clinical specimens.

RAPD PCR

This method is fast and easy to perform and all strains could be differentiated at once. RAPD was used to explore the unique binding pattern of two different strains used in the study. For this random primer OPAP-01 was used and the banding patterns obtained with the two cultures were quite distinct and good enough to discriminate them as two separate strains. The typical banding patterns of standard and test cultures using primer OPAP-01 have been projected in Fig.2**.** Similar results were observed by Johansson *et al* 1995 in *L.plantarum* strains. Torriani *et al* 2001 used RAPD PCR for differentiation of *Lactobacillus plantarum*, *L. pentosus* and *L. paraplantarum* and observed species-specific electrophoretic profiles. Cocconcelli *et al* 1997 observed that RAPD and 16S rDNA sequencing to be an efficient tool for the analysis of *Lactobacillus* populations in natural whey cultures. Thus the approaches to the detection and identification of *Lactobacillus* species that we have described in this report will contribute to future studies of the composition of the intestinal microflora and to a better understanding of *Lactobacillus* taxonomy.

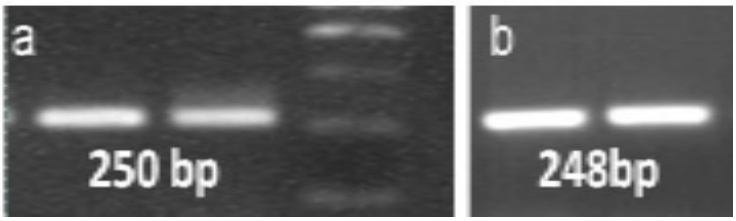
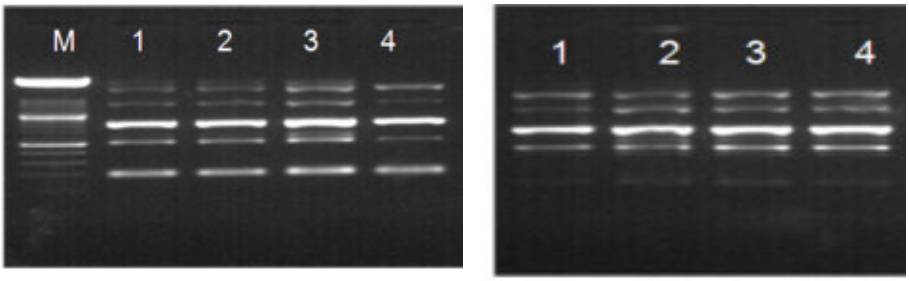


Figure 1
Genus and Species specific PCR using primer LpLmlA1 /161R1 and Lpla3/Lpla2.
(a) Lane 1: Lp91, Lane 2: Lp5276 ,Lane 3: Marker 100bp(Genus specific PCR)
(b) Lane 1: Lp91, Lane 2: Lp5276 (Species specific PCR)



(a) **(b)**

Figure 2
RAPD profiles of lactobacillus cultures using primer OPAP-01
a:M- Marker,Lane1,2,3,4 Lp 91 ; b: Lane1,2,3,4 Lp5276.

CONCLUSION

In this study the identification of *Lactobacillus plantarum* strains were done using PCR and RAPD. When compared to the conventional techniques for identification this will serve as a rapid method of identification as well as differentiation of different bacterial strains. This will be certainly useful for easy selection of lactic acid bacteria for preparing fermented dairy products and probiotic foods.

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